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Direct somatic embryogenesis with high frequency plantlet regeneration and successive cormlet production in saffron (*Crocus sativus* L.)

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ABSTRACT

Saffron is a slowly propagating geophyte with fungal infestation of corms under field conditions. Moreover, genetic improvement through breeding is not possible due to male sterility. Therefore, tissue culture methods offer a great potential for mass propagation, and somatic embryogenesis is one of the efficient methods. In the present study, direct somatic embryogenesis from leaf base was obtained in thidiazuron (TDZ, 2.5 μM) and picloram (2.0 μM) supplemented Murashige and Skoog medium (Murashige and Skoog, 1962). Significantly higher secondary embryogenesis was observed in embryo proliferation medium (EPM; MS supplemented with TDZ 2.5 μM and picloram 1.0 μM). The origin and different developmental stages of somatic embryos were ascertained through histological and scanning electron microscopic studies. Stomata were also observed in some of the somatic embryos. An alluring observation was retention of embryogenic potential beyond 3 years of culture. Percent germination of secondary embryos in MS medium was 60.48 when matured in ½ EPM. Freshly initiated somatic embryos also germinated to form plantlets without undergoing secondary embryogenesis in MS medium containing TDZ (2.5 μM) and picloram (1.0 and 2.0 μM). Somatic embryo derived shoots were multiplied in 6-benzylaminopurine (BAP; 26.64 μM) and α-naphthaleneacetic acid (NAA; 1.0 μM). Growth performance of cormlets obtained from these shoots was evaluated under green house conditions. Somatic embryogenesis holds tremendous importance for mass propagation of saffron with formation of ultimate propagating material i.e., cormlets.

1. Introduction

Crocus sativus L. (family Iridaceae), a male sterile, self incompatible geophyte with no seed set, is propagated through corms. It produces one or two bisexual flowers in one growing season and only the orange red tripartite stigma with part of style produces the spice saffron. In conventional methods crop improvement is restricted to evaluation, selection and vegetative propagation of naturally occurring or artificially induced clonal variants. Moreover, large scale production of daughter corms is a long drawn process. The mother corm perishes after forming 4–5 replacement corms. Besides, the latent endogenous infection in mother corms infects daughter corms and thereby affects the productivity. In saffron, spice yield is dependent upon flowering which is directly related to corm size and health. Application of tissue culture methods offers great potential in overcoming these challenges for production of new varieties, mass multiplication of selected ecotypes or improved varieties and genetic improvement.

Somatic embryogenesis and subsequent regeneration into plantlets is a striking alternative for cormlet production and large scale propagation in saffron. The cormlets produced through tissue culture can be used as planting material in conventional method of propagation to increase productivity and decrease disease incidence. Few reports are available on in vitro cormlet formation through somatic embryo derived shoots but with an intermediate callus formation (Ahuja et al., 1994; Karamian, 2004; Raja et al., 2007; Sheibani et al., 2007) and viable protocol is unavailable. Moreover, in vitro produced cormlets were not evaluated for growth performance under in vivo conditions, which is imperative for success of a micro-propagation protocol.

Present study was aimed at direct somatic embryogenesis using leaf bases as explants, cormlets production and growth performance evaluation of in vitro cormlets under green house conditions. In order to ascertain the different developmental stages histological and scanning electron microscopic studies were carried out. The effect of reserve accumulation and amylase activity during different developmental stages was also studied.

2. Materials and methods

2.1. Induction of somatic embryos

Corms procured from natural habitat (Pampore, Srinagar, J&K, India, 34°1′12″ N and 74°55′5″ E at 1574 m amsl) were surface sterilized

Abbreviations: EPM, embryo proliferation medium; NRS, non-reducing sugars; PBZ, paclobutrazol; RS, reducing sugars; TSS, total soluble sugars.

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following the standardized protocol (Devi et al., 2011) and inoculated on MS (Murashige and Skoog, 1962) basal medium. Once the buds sprouted (both apical and axillary), young true leaves (5.0–6.0 cm) which were still inside the leaf sheath (cataphylls), were carefully separated, cut into segments (2.0–2.5 cm, Fig. 1a) and inoculated on MS medium supplemented with TDZ (1.0, 2.5 and 5.0 μM) and picloram (0.5, 1.0 and 2.0 μM) in factorial combinations. Medium without PGRs served as control. Three leaves per replicate (Petriplate, 90 mm, Tarsons, India) and five replicates per treatment were used. Data in terms of percent response of swelling of leaf base and number of somatic embryos formed were recorded after 4, 8 and 12 wks of culture. Cultures were maintained at 25 ± 2 °C under 16/8 h of light and dark cycle with photosynthetic photon flux density (PPFD) of 70 ± 5 μmol m^{-2} s^{-1}. In all the experiments, culture conditions were the same unless otherwise specified.

2.2. Proliferation of somatic embryos

2.2.1. Effect of TDZ and picloram

TDZ (1.0, 2.5 and 5.0 μM) and picloram (0.5, 1.0 and 2.0 μM) were used in factorial combinations. In all the experiments uniform samples i.e., clumps of somatic embryos (1.0 g each) per flask (250 ml Erlenmeyer, Borosil, India) were inoculated on medium and five replicates per treatment were used. Data were recorded after 6 wks of incubation.

2.2.2. Effect of light conditions

Embryo proliferation medium (EPM; MS medium supplemented with TDZ 2.5 μM and picloram 1.0 μM) used was derived from the above experiment. Uniform sample per flask was used as initial explants. Activated charcoal (AC, 1.0%, w/v) and ascorbic acid (0.1%, w/v) was added to check phenolic exudations. Four replicates per treatment were taken in further experiments. Data were recorded after 6 wks of incubation. Cultures were incubated at 25 ± 2 °C in continuous dark and light/dark cycle of 16/8 h with PPFD 70 ± 5 μmol m^{-2} s^{-1}.

2.2.3. Effect of temperature

Clumps of somatic embryos on EPM were incubated in the dark at 10, 15, 20 and 25 ± 2 °C. Data were recorded after 6 and 10 wks of culture and the parameters studied were increase in biomass i.e., fresh weight of cultures and growth index. Growth index (GI) was calculated by using following formula

$$GI = \frac{FW_f - FW_i}{FW_i} \times 100$$

where

FW_i initial fresh weight
FW_f final fresh weight

2.3. Maturation and conversion of somatic embryos

2.3.1. Effect of ABA and GA_3

Clumps of somatic embryos were transferred to MS medium supplemented with ABA (5.67 and 11.34 μM) and GA_3 (28.9 and 57.8 μM) in factorial combinations. Three replicates per treatment were used and data recorded after 8 wks of culture.

In another set of experiments, EPM supplemented with ABA (1.89, 3.78, 7.56 and 15.12 μM) was used for maturation of somatic embryos. Medium without ABA served as control. Clumps containing ~150 somatic embryos per replicate were used. After 4 wks of culture on respective media with different ABA concentrations, somatic embryos were transferred to MS medium without PGRs or fortified with GA_3 (conversion medium) at concentrations of 14.95, 28.9 and 57.8 μM. Data were recorded after 8 wks of transfer of somatic embryos from maturation to conversion medium.

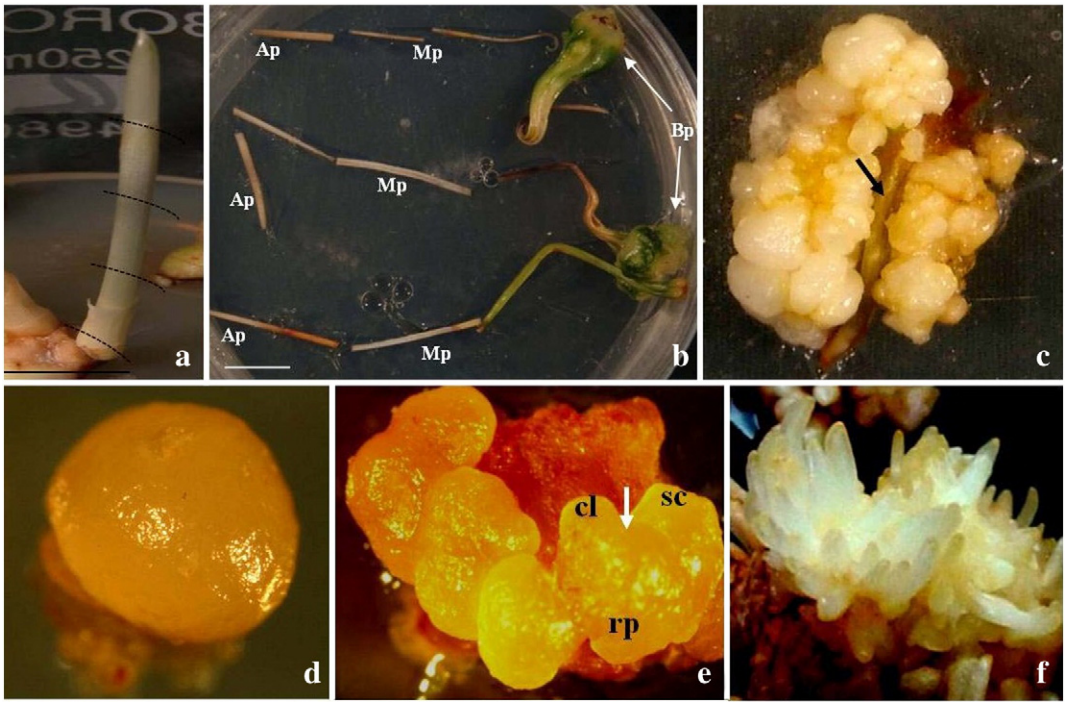

Fig. 1. Somatic embryo induction a) Bud sprouts used for somatic embryo induction (lines indicate the plane of cutting); b) swelling of basal portion of leaf (arrow) (Bp = basal, Mp = middle, Ap = apical portion of leaf); c) induction of somatic embryogenesis from swollen basal portion of leaf in picloram (2.0 μM) and TDZ (2.5 μM) (arrow indicates leaf explant); d) globular somatic embryo; e) somatic embryo with a notch (arrow) showing coleoptile (cl), scutellum (sc) and root pole (rp); bar line = 1.0 cm; f) somatic embryo derived multiple shoots.

2.3.2. Effect of sucrose

2.3.2.1. Full strength media (EPM and MS) and sucrose. EPM and MS supplemented with varying concentrations of sucrose (1.0, 3.0, 6.0, 9.0 and 12.0%) were used for maturation and conversion of somatic embryos. Culture conditions were the same as described in Section 2.2.2. Data were recorded after 14 wks of culture.

2.3.2.2. Half strength media (EPM and MS) and sucrose. Half strength EPM (½ EPM) and MS (½ MS) media and different concentrations of sucrose (1.0, 3.0, 6.0, 9.0 and 12.0%) were used. After 6 wks of incubation in the dark at 25 ± 2 °C, somatic embryos were transferred to MS basal medium for germination. Percent germination was recorded after 8 wks of culture.

2.4. Retention of biomass proliferation and embryogenic potential

Clumps of somatic embryos differentiated during different time intervals [approx. four and a half year old culture (4½ years), approx. three and a half year old culture (3½ years), approx. two year old culture (2 years) and a one year old culture (1 year)] were taken. Cultures in EPM were incubated in the dark at 25 ± 2 °C, as incubation in the light led to phenolic exudations. Data for fresh weight were recorded after 6 wks of incubation. For calculating dry weight, somatic embryos were dried at 60 °C till the weight of somatic embryos becomes stable. Percent moisture content was calculated using the formula

$$\text{Moisture } \% = \frac{FW - DW}{FW} \times 100$$

where

FW fresh weight
DW dry weight

$$\text{Final } \% \text{ moisture content} = S1 + S2\text{-}\frac{S1 \times S2}{100}\%$$

where

S1 percent moisture content from first stage of drying
S2 percent moisture content from second stage of drying.

2.5. Cormlet production from somatic embryo derived shoots and growth performance under greenhouse conditions

Somatic embryo generated shoots were cultured in MS medium supplemented with 6-benzylaminopurine (BAP; 26.64 μM) and α-naphthaleneacetic acid (NAA; 1.0 μM) for shoot multiplication. These multiple shoots were then used for cormlet production in medium containing paclobutrazol (PBZ; 1.7 μM). Cormlet production was carried out at 15 ± 2 °C and continuous light (PPFD 38 μmol m^{-2} s^{-1}) (Devi et al., 2011).

In vitro cormlets (somatic embryo derived) were divided in categories of 0.1–1.2 g on the basis of weight. Growth performance was evaluated in pots using soil:sand:FYM in a ratio of 1:2:1 under green house conditions (temperature, 20–30 °C and relative humidity, 60–70%). Data recorded were number of sprouts, number of leaves and leaf length after 5 months of transfer. Fifteen cormlets for each category were used.

2.6. Histological studies

Somatic embryos at different developmental stages were fixed in formalin/acetic acid/50% ethanol (1:1:18; v/v/v) and dehydrated in tertiary butyl alcohol series (Ruzin, 1999). Material was embedded in paraffin wax and 12 μm thick sections were cut using a rotary microtome (Shandon Finesse, Thermo Electron, ME, USA). Sections were stained with safranin and fast green and observed under a stereozoom microscope (Nikon SMZ1500, Japan).

2.7. Scanning electron microscopic studies

Somatic embryos were frozen in liquid nitrogen for 2 to 5 min, mounted on aluminium stubs using double adhesive tape, coated with carbon/gold using sputter coating unit (E-1010, Hitachi, Japan) and observed under Scanning Electron Microscope (S-3400 N Hitachi, Japan).

2.8. Biochemical studies

Biochemical analysis of somatic embryos was done to see the effect of different concentrations of ABA, sucrose and medium strength on reserve accumulation. Amylase assay was done using DNS (3,5-dinitrosalicylic acid, Bernfield, 1995). Starch was measured as simple sugars (glucose), liberated as breakdown products using anthrone reagent (Adams et al., 1980). Glucose content was calculated using standard curve and then the value was multiplied by factor of 0.9 to arrive at the starch content. TSS (total soluble sugars) was measured by phenol-H_2SO_4 method (Dubois et al., 1956). It works on the principle that in hot acidic medium glucose is dehydrated to hydroxyl methyl furfural and forms a green colored product with phenol. RS (reducing sugars) was measured using the DNS (Miller, 1959). NRS (non-reducing sugars) were calculated by subtracting the value calculated for RS from TSS.

2.9. Statistical analysis

Experiments were designed employing completely randomized design. All the statistical analysis was performed using Statistica data analysis software (STATISTICA release 7, Statsoft Wipro, India) with number of factors described separately in different headings for various experiments. Values were subjected to analysis of variance (ANOVA), employing the general linear model for main-effect ANOVA. The statistical significance between the mean values was assessed by applying Duncan's Multiple Range Test (DMRT). A probability level of $P \leq 0.05$ was considered significant. Values in the tables are followed by standard error (value ± SE). Percent data was subjected to square root transformation and denoted by * sign. SEM is standard error of means of all the populations.

3. Results

3.1. Induction of somatic embryos

Swelling at the base of the leaf (meristematic region) was observed after 4 wks of culture in MS medium supplemented with varying concentrations of TDZ and picloram (Fig. 1b). Response was 100% in TDZ 1.0 μM and picloram 2.0 μM (Table 1). Middle (Mp) and tip (Ap) portions of the leaves remained green for 3–4 wks but turned necrotic later on (Fig. 1b). Well organized, cream colored, globular somatic embryos appeared at the swollen basal portion (Bp) of leaf without intervening callus phase, after 8 wks of culture in medium supplemented with picloram (2.0 μM) and all the concentrations (1.0, 2.5 and 5.0 μM) of TDZ (Table 1; Fig. 1c). Significantly higher mean number of somatic embryos (30.11) was formed in picloram (2.0 μM) and TDZ (2.5 μM) after 12 wks of culture (Table 1). Medium supplemented with lower concentrations of picloram (0.5 and 1.0 μM) and all the concentrations of TDZ were late responding and somatic embryogenesis was induced after 8 wks of culture. Somatic embryos were tightly packed and inseparable. Such somatic embryos were separated from initial explants and sub-cultured to fresh medium for proliferation. No response was evident when TDZ and picloram were used singly.

Table 1
Induction of somatic embryogenesis from in vitro leaves on TDZ and picloram containing medium.

TDZ (μM)	Picloram (μM)	% Swelling response	Mean number of embryos after	
		4 wks	8 wks	12 wks (SEM = 4.62)
0	0	0	NR	–
0	0.5	0	NR	–
0	1.0	0	NR	–
0	2.0	0	NR	–
1.0	0	0	NR	–
1.0	0.5	27.78 (26.75)*	NR	6.44 ± 0.59^{g}
1.0	1.0	44.33 (41.69)	NR	12.20 ± 1.15^{e}
1.0	2.0	100.00 (90.00)	8–10	20.22 ± 1.35^{b}
2.5	0	0	NR	–
2.5	0.5	66.00 (54.33)	NR	15.00 ± 0.59^{d}
2.5	1.0	88.67 (78.11)	NR	17.10 ± 1.16^{c}
2.5	2.0	88.67 (78.11)	10–12	30.11 ± 2.21^{a}
5.0	0	0	NR	–
5.0	0.5	44.43 (41.75)	NR	9.30 ± 0.88^{f}
5.0	1.0	71.33 (67.32)	NR	14.56 ± 1.39^{d}
5.0	2.0	86.67 (76.92)	9–10	17.76 ± 1.2^{c}

Data represented as the means of 5 replicates; lower case letters differ from each other at $p \leq 0.05$ (Duncan's multiple range test); data recorded after 4, 8 and 12 wks.
* Transformed values; NR — No response.

3.2. Proliferation of somatic embryos

3.2.1. Effect of TDZ and picloram

Significant increase in biomass (1.99 g) and mean number of somatic embryos (297.75) were observed in picloram (1.0 μM) and TDZ (2.5 μM) (Table 2). Increased concentration of TDZ (5.0 μM) in combination with picloram (0.5, 1.0 and 2.0 μM) resulted in reduction in biomass (0.19, 0.88 and 0.49 g, respectively) and number of somatic embryos, which turned necrotic afterwards. White or cream colored somatic embryos underwent secondary embryogenesis to form clumps of tightly packed somatic embryos. Globular somatic embryos with soft and shining surface (Fig. 1d) grew further to form somatic embryo with a notch that differentiated to form scutellum, coleoptile, root and shoot pole (Figs. 1e, 2a). In some of the somatic embryos, stomata were also observed (Fig. 2b). Scanning electron microscopic studies indicated that surface cells of globular embryos were smooth and regularly arranged while that of coleoptilar embryos were reticulated in nature. Sub-culturing at 4 wks interval in TDZ (2.5 μM) and picloram (1.0 μM) resulted in vigorous proliferation of somatic embryos.

3.2.2. Effect of light and dark conditions

No significant difference in proliferation of somatic embryos was observed under light (4.27 g fw) and dark (3.95 g fw) conditions but phenolic exudations were very high under light conditions. Somatic embryos turned into hard structures and growth hampered when activated charcoal was added, whereas bleaching and death occurred in ascorbic acid. Therefore, dark conditions were found suitable for proliferation of somatic embryos.

Table 2
Proliferation of somatic embryos in TDZ and picloram containing medium.

Picloram (μM)	TDZ (μM)	Increase in biomass (mean) after 6 wks (g) (SEM = 0.094)	Mean no. of somatic embryos (SEm = 21.4)
0.5	1.0	1.70 ± 0.17^{ab}	204.0 ± 3.18^{bc}
0.5	2.5	1.20 ± 0.13^{bcd}	180.0 ± 5.77^{bc}
0.5	5.0	0.19 ± 0.07^{f}	28.5 ± 1.73^{e}
1.0	1.0	1.48 ± 0.10^{abc}	222.0 ± 14.26^{ab}
1.0	2.5	1.99 ± 0.16^{a}	297.8 ± 17.04^{a}
1.0	5.0	0.88 ± 0.26^{de}	132.5 ± 15.86^{cd}
2.0	1.0	1.15 ± 0.11^{bcd}	172.0 ± 4.32^{bc}
2.0	2.5	1.02 ± 0.08^{cde}	153.5 ± 5.78^{bcd}
2.0	5.0	0.49 ± 0.34^{ef}	73.0 ± 2.52^{ce}

Initial wt. of clumps of somatic embryos = 1.0 g; data were recorded after 6 wks; data represented as the means of five replicates/treatment; values followed by lower case letters differ from each other at $p \leq 0.05$ (Duncan's multiple range test).

3.2.3. Effect of temperature

Somatic embryo proliferation increased with increasing temperature. Effect of temperature was studied only up to 25 ± 2 °C as saffron cultures do not grow beyond this temperature range. At 25 ± 2 °C, biomass (8.29 g) was significantly higher after 10 wks (Table 3) and the highest GI was observed. Lower temperature was not suitable for the proliferation of somatic embryos as these got deformed and turned necrotic at 10 ± 2 °C.

3.3. Maturation and conversion of somatic embryos

3.3.1. Effect of ABA and GA_3

Low germination (2.0–3.0%) was observed in MS medium supplemented with ABA (5.67 and 11.34 μM). In medium fortified with GA_3 (28.9 and 57.8 μM) bleaching of cultures was observed that ultimately resulted in the death of somatic embryos. When ABA (5.76 μM) was used in combination with low concentration of GA_3 (28.9 μM), low germination percent was observed but in higher concentration of ABA (11.34 μM) in combination with both concentrations of GA_3, death of somatic embryos was observed without any conversion (data not given).

Conversion of somatic embryos was also carried out in a two step procedure with maturation in ABA and germination in GA_3 containing medium. In maturation medium, somatic embryos turned into compact and soft structures after 4 wks of culture. Prior culture of somatic embryos for 6 wks in ABA (7.56 μM) containing medium and transfer to MS basal medium or GA_3 (14.45 μM) containing medium evinced 27.33 and 30.0% germination, respectively (Table 4). Number of germinated somatic embryos was also higher (45) in latter medium combination. Decline in germination was observed with increasing concentrations of both ABA and GA_3. Although conversion of somatic embryos was achieved, yet shoots formed were thin and tender and did not resume growth.

3.3.2. Effect of sucrose

3.3.2.1. Full strength media (EPM and MS) and sucrose. Somatic embryos proliferated well in 3.0% sucrose in both media under light/dark (16/8 h) and dark conditions but no germination was observed. In EPM, somatic embryos underwent recurrent somatic embryogenesis but in MS basal medium, a little callusing was observed. At 6.0% sucrose and beyond, nodular and hard structures were formed which did not proliferate in both light/dark (16/8 h) and dark conditions. After 14 wks of culture in EPM with 12.0% sucrose, somatic embryos germinated but did not grow further to form plantlets under dark conditions. No germination occurred in other medium combinations even after 6 wks of culture at 25 ± 2 °C and 16/8 h light and dark conditions. Therefore, in another set of experiment, medium strength was reduced to half and experiment performed under dark conditions.

3.3.2.2. Half strength media (EPM and MS) and sucrose. Concentration of sucrose in the maturation medium showed marked effect on increase in biomass and percent germination of somatic embryos. Increase in fresh weight was significantly high in ½ EPM (2.82 g) with 3.0% sucrose. Somatic embryos turned into hard nodular structures in both maturation media used. Increase in sucrose concentration to 6.0% or above resulted in decrease in biomass and proliferation of somatic embryo. In 1.0% sucrose somatic embryos turned necrotic and neither proliferation nor maturation was observed. Percent germination was highest in somatic embryos matured in ½ EPM (60.48%) (Table 5; Fig. 1f). Percent germination response decreased with increasing sucrose concentration in maturation medium. The germinated somatic embryos formed bunch of shoot buds (Fig. 1f) which further grew into multiple shoots in BAP

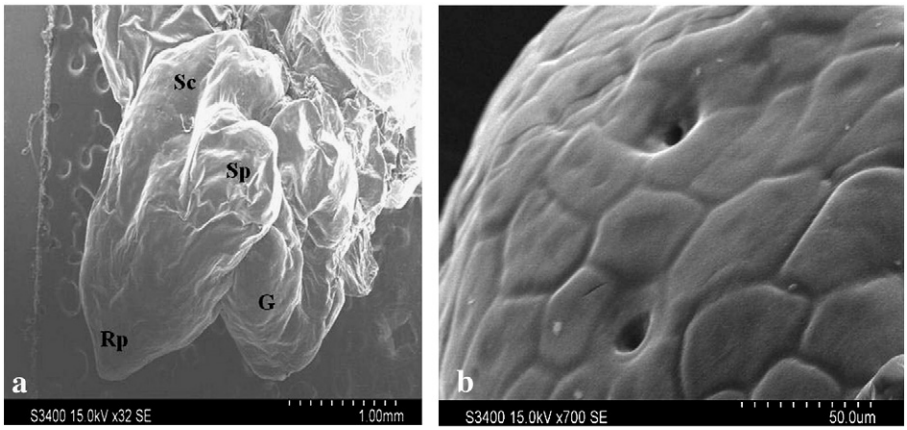

Fig. 2. Scanning electron micrographs of somatic embryos a) somatic embryos at different developmental stages (G — globular embryo, Sp — shoot pole, Sc — scutellum, Rp — root pole); b) stomata on surface of somatic embryos.

(26.64 μM) and NAA (1.0 μM) supplemented medium. Development of root and shoot simultaneously was infrequently observed. Somatic embryos matured in half strength MS medium did not germinate.

3.4. Seasonal effect on germination of somatic embryos

Somatic embryos maintained in EPM, during a particular time period i.e., January to April formed shoots when left in same medium for 6–8 wks without sub-culturing. Conversion percent was about 10.0–15.0. These shoots formed multiple shoots when sub-cultured on standardized shoot multiplication medium i.e., MS medium with BAP (26.64 μM) and NAA (1.0 μM).

Occasionally freshly initiated somatic embryos did not undergo secondary embryogenesis and germinated in the same medium i.e., in MS medium containing TDZ (2.5 μM) and picloram (1.0 and 2.0 μM). Such somatic embryos formed only shoots but no roots and germination percent was low (data not shown).

Germination potential of somatic embryos maintained in EPM was observed to be more than three years. Three and a half and 2 year old somatic embryos germinated in the month of March in EPM to form shoots. Secondary embryogenesis was also evident in these cultures. Four and a half year old somatic embryos did not germinate to form shoot or root.

3.5. Retention of biomass proliferation and embryogenic potential

Significant increase in cell biomass (fresh weight, 3.77 g) was observed after 6 wks in 4½ year old cultures (Fig. 3). Fresh weight of 3½ year and 2 year old somatic embryos were at par, whereas 1 year old cultures showed low proliferation rate which increased eventually with time and was consistent after two years. Relative growth rate was comparable in all the samples but growth index was lowest in 1 year old cultures. Percent moisture content of somatic embryos of all durations was comparable (99.03–99.27). SEM studies revealed that 4½ year and 3½ year old cultures showed callusing along with somatic embryos. However, the extent of callusing was more in the former. In the 2 year and 1 year old cultures no callusing was observed.

3.6. Cormlet production from somatic embryo derived shoots

Cormlets with fully developed tunic were obtained after 12–14 wks of culture (Fig. 4a, Devi et al., 2011). In vitro produced cormlets (Fig. 4b) were comparable to the naturally produced corms (Fig. 4c).

3.7. Growth performance evaluation

Under greenhouse conditions cormlets derived from somatic embryo generated shoots revealed maximum percent sprouting (89.0) and significantly high mean number of leaves (2.0) in biggest sized (1.0–1.2) cormlets. Mean number of sprouts was significantly high (2.25) in 0.7–0.8 g of cormlets (Table 6).

3.8. Histological studies

Histological studies were carried out to ascertain different developmental stages of somatic embryos. Fig. 5a shows a transverse section of leaf at day zero where vascular bundles were regularly arranged. After 4 wks, the leaf swelled on picloram (2.0 μM) and TDZ (2.5 μM) supplemented medium (Fig. 5b). Vascular bundles were scattered but fused throughout the swollen leaf (Fig. 5c). Cells enlarged in size and periclinal or anticlinal cell divisions occurred in both epidermal (Fig. 5d) and hypodermal (Fig. 5e) cells. Groups of densely cytoplasmic and small meristematic cells with distinctive nucleus were formed at the peripheral region of the leaf. As a result of cell division, small pockets of 4–6 cells (Fig. 5e) enclosed by a common cell wall were formed. The protuberances were comprised of densely cytoplasmic cells (Fig. 5f) which further developed into globular embryos (Fig. 5g) covered with a well defined protodermis. The compartmentation indicates the unicellular origin of these proembryo-like structures.

Shoot apex and radical end of somatic embryo contained densely cytoplasmic cells with deeply stained nuclei. Independent vascular strand developed in these somatic embryos. Primordium of somatic embryo grew as an outgrowth and a notch appeared at the terminal position (Fig. 5h). A hump appeared near the notch where the shoot apex is

Table 3
Effect of temperature on somatic embryo proliferation.

Duration	Parameters	10 ± 2 °C	15 ± 2 °C	20 ± 2 °C	25 ± 2 °C
6 wks	FW (g)	1.60 ± 0.03^{c}	1.94 ± 0.34^{c}	3.00 ± 0.36^{b}	3.83 ± 0.13^{a}
	GI	37.40 ± 2.12	48.45 ± 8.44	66.67 ± 8.28	73.86 ± 1.86
10 wks	FW (g)	2.36 ± 0.27^{b}	4.32 ± 0.80^{b}	5.42 ± 1.04^{ab}	8.29 ± 0.78^{a}
	GI	46.17 ± 0.93	65.97 ± 4.14	80.50 ± 4.22	116.64 ± 1.06

Data represented as the means of 4 replicates; data recorded after 6 and 10 wks; values followed by lower case letters differ from each other at $p \leq 0.05$ (Duncan's multiple range test).

Table 4
Maturation and conversion of somatic embryos.

Maturation medium EPM + ABA (μM)	Germination medium MS + GA_3 (μM)	Mean number of germinated embryos (SEm = 10.66)	Percent germination response
0	–	7.67^{e}	5.11
1.89	–	10.33^{de}	6.67
3.78	–	–	–
7.56	–	41.0^{ab}	27.33
15.12	–	15.0^{d}	10
0	14.45	21.0^{c}	10.22
1.89	14.45	–	–
3.78	14.45	–	–
7.56	14.45	45.0^{a}	30.0
15.12	14.45	–	–
0	28.9	–	–
1.89	28.9	–	–
3.78	28.9	–	–
7.56	28.9	37.5^{b}	25.0
15.12	28.8	15.0^{d}	10.0
0	57.8	–	–
1.89	57.8	–	–
3.78	57.8	–	–
7.56	57.8	–	–
15.12	57.8	10.0^{de}	10

EPM — Embryo proliferation medium [(MS + TDZ (2.5 μM) + picloram (1.0 μM)]; data represented as the means of 4 replicates; data recorded after 8 wks; values followed by lower case letters differ from each other at $p \leq 0.05$ (Duncan's multiple range test).

organized. The shoot apex had lateral position and the root apex developed opposite to the shoot apex. Somatic embryo developed further to form shoot pole (coleoptile), root pole (coleorhiza) and scutellum with independent vascular system (Fig. 5i). Since in saffron the corm develops prior to the root, the tissue destined to form corm was visible. Germinated somatic embryo formed shoot apex, root apex, prophyll, roundish corm and independent vascular bundle (Fig. 5j). Some of the somatic embryos had single root and corm primordium and number of shoot primordia (Fig. 5k) with connecting vascular bundles between shoot and root poles. At the base of germinating somatic embryo, secondary embryogenesis was also observed (Fig. 5l). Even though integrated shoot, root and corm apex appeared in somatic embryos but simultaneous development did not appear. Somatic embryo showed mono-polarity and shoots multiplied further in shoot multiplication medium.

3.9. Biochemical studies

3.9.1. Effect of different concentrations of ABA on reserve accumulation

3.9.1.1. Amylase. As the treatment duration progressed, the amylase activity decreased uniformly in somatic embryos matured in ABA supplemented EPM. It was higher after 15 days (0.28–0.30 mg g^{-1} fw) of incubation and decreased after 30 days (0.25–0.28 mg g^{-1} fw) (Table 7). No significant difference in amylase was observed in different concentrations of ABA in the medium.

3.9.1.2. Total reserve accumulations

3.9.1.2.1. Starch and sugars. Starch content increased with increasing concentration of ABA and was maximum in 7.56 μM after 15 (4.40 mg g^{-1} fw) and 30 (1.93 mg g^{-1} fw) days of incubation. It started decreasing with further increase in concentration of ABA (Table 7). TSS content also increased with increasing concentration of ABA. After 15 days of treatment, TSS was highest in somatic embryos treated with 7.56 μM of ABA. Almost the same trend was observed after 30 days of incubation. The major part of total TSS was constituted by NRS and was higher in 7.56 μM of ABA after 15 days (29.86 mg g^{-1} fw) and 30 days (34.64 mg g^{-1} fw). Both starch and sugar contents were lowest in control.

3.9.2. Biochemical analysis of somatic embryos matured in ½ EPM

Amylase activity was highest in matured somatic embryos compared to proliferating secondary somatic embryos and was lowest in germinated somatic embryos. Starch content was low (3.97 mg g^{-1} fw) in secondary somatic embryos cultured in EPM but increased in matured somatic embryos (5.51 mg g^{-1} fw) cultured in ½ EPM for 6 wks and further decreased (4.83 mg g^{-1} fw, in MS basal medium) in early germinated somatic embryos (Table 8). The content of total soluble sugars was highest in matured somatic embryos (38.5 mg g^{-1} fw) and was low in proliferating somatic embryos (23.09 mg g^{-1} fw) and freshly germinated ones (11.18 mg g^{-1} fw). Major part of sugar was constituted by non reducing sugars and was maximum (34.62 mg g^{-1} fw) in matured somatic embryos. Reducing sugar was low in comparison to NRS and was highest in secondary proliferating somatic embryos in comparison to matured and germinated somatic embryos.

4. Discussion

In in vitro recalcitrant plants, usually juvenile parts are responsive to somatic embryogenesis or regeneration (Schulze, 2007; Stasolla et al., 2002). In saffron also vegetative apex (George et al., 1992), apical buds (Blázquez et al., 2004), shoot meristem along with a pair of leaf primordia (Ahuja et al., 1994; Karamian, 2004), rectangular section from central meristematic region (Sharifi et al., 2010) were used as explant for somatic embryogenesis. In the present study, direct somatic embryogenesis was initiated from the leaf base (Fig. 1b) which is a site of meristematic activity in monocots, in picloram (2.0 μM) and TDZ (2.5 μM) supplemented medium (Fig. 1b; Table 1). Use of TDZ for somatic embryogenesis is well documented in many plants (Guo et al., 2011). In saffron, Vatankhah et al. (2010) obtained organogenic callus from shoot meristem in kinetin (4.0 μM) and picloram (10.0 μM) or TDZ (2.0 μM) and NAA (1.0 μM) under dark conditions. Auxins and

Table 5
Effect of sucrose concentration on germination of somatic embryos in half strength media.

Maturation medium	Sucrose concentration (%)	Increase in fresh weight after 6 wks (g) (SEm = 0.80)	Mean no. of somatic embryos	Germination medium	Percent germination after 8 wks
½ MS	1.0	0.08 ± 0.08^{c}	–	MS basal medium with 3.0% sucrose	–
	3.0	2.16 ± 0.69^{ab}	–		–
	6.0	1.46 ± 0.87^{abc}	–		–
	9.0	1.38 ± 0.44^{ab}	–		–
	12.0	0.61 ± 0.23^{bc}	–		–
½ EPM	1.0	0.19 ± 0.08^{c}	28		–
	3.0	2.82 ± 0.26^{a}	420		60.48
	6.0	2.12 ± 0.62^{ab}	373.5		53.82
	9.0	1.52 ± 0.64^{abc}	228		40.35
	12.0	1.54 ± 0.53^{abc}	231		30.74

EPM — Embryo proliferation medium; data represented as the means of 4 replicates; data recorded after 6 and 8 wks of transfers to maturation and germination medium respectively; values followed by lower case letters differ from each other at $p \leq 0.05$ (Duncan's multiple range test).

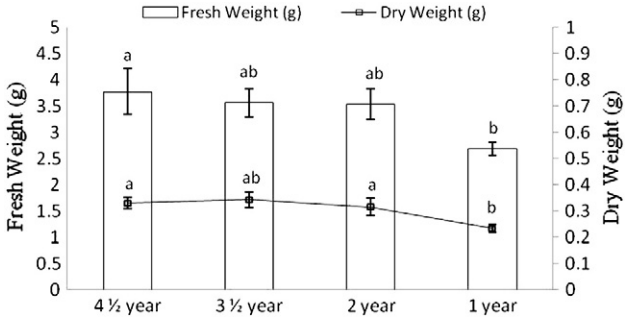

Fig. 3. Fresh weight and dry weight of embryos generated at different time periods.

cytokinins due to strong participation in cell cycle regulation and cell division are the key factors to determine the embryogenic response (Francis and Sorrell, 2001). It was postulated that the concentration of endogenous phytohormones affected TDZ induced morphogenesis (Guo et al., 2011). TDZ increased the supply of purines for cellular development and accumulation of purine metabolites (Victo et al., 1999). These are required during cell division and protein synthesis which are very fast during somatic embryo development (Fujimura and Komamine, 1980). Significantly higher secondary embryogenesis was observed on TDZ (2.5 μM) and picloram (1.0 μM) containing medium (Table 2). In higher concentration of TDZ, proliferation rate declined with browning and death of somatic embryos as has earlier been reported in *Capsicum annuum* (Khan et al., 2006).

Light and dark conditions did not show any significant difference in proliferation and morphology of somatic embryos but dark conditions minimized the phenolic exudations. Somatic embryo proliferation at 25 ± 2 °C (Table 3) and 16/8 h light and dark cycle is in agreement with the previous reports on saffron (Darvishi et al., 2006; Ebrahimzadeh et al., 2000; Karamian, 2004; Rajabpoor et al., 2007; Vatankhah et al., 2010).

Maturation of somatic embryos is a vital juncture of embryogenesis for getting high frequency of healthy plants. In the present study, somatic embryo germination was achieved only when maturation and germination were performed as two step procedure i.e., EPM medium with ABA for maturation and MS medium with or without GA_3 for conversion (Table 4). Literature reports on saffron indicate that maturation of somatic embryos was carried out in ABA (George et al., 1992; Karamian, 2004) and germination in medium devoid of PGRs (Sheibani et al., 2007) or supplemented with GA_3 (Ahuja et al., 1994; Ebrahimzadeh et al., 2000; Karamian, 2004). Raja et al. (2007) combined steps of maturation and germination by using medium supplemented with ABA (1.75 μM) along with BAP (0.5 μM) and GA_3 (20.0 μM). Somatic embryos were also matured in half strength MS medium without any additive (Ahuja et al., 1994; Ebrahimzadeh et al., 2000). Picloram with other PGRs is also known to regulate the embryogenic stages and produced maximum frequency of somatic embryos and germination (Ahmed et al., 2011; Little et al., 2000). According to George and Eapen (1993) conversion of somatic embryos into plantlets depends on the type and concentration of auxin used for induction of somatic embryos. Darvishi et al. (2006) used LS medium supplemented with BAP (8.88 μM) and 2,4-D (9.05 μM) for induction, maturation and germination of somatic embryos in saffron.

Concentration of sucrose in the maturation medium showed marked effect on increase in biomass and percent germination of somatic embryos. Sucrose may act as a source of carbon, energy and osmotic agent (Carrier et al., 1997). Percent germination was highest (60.48) in somatic embryos matured in ½ EPM with 3% sucrose (Table 4) and germinated in MS basal medium but decreased with further increase in sucrose concentration. Earlier in saffron, somatic embryos generated in TDZ containing medium were matured in MS medium devoid of PGRs with 6.0% sucrose at 4 °C and germinated in MS basal medium (Sheibani et al., 2007) or sucrose (4.5%) coupled with 2.38 μM jasmonic acid (Blázquez et al., 2004). Sucrose probably substituted the starch reserves, whereas decrease in percent germination response at higher concentrations of sucrose could be due to osmotic shock (Mondal et al., 2002).

During a definite time period i.e., January to April conversion of proliferating somatic embryos on EPM without sub-culturing for 6–8 wks indicated that somatic embryos, like shoot primordia, probably followed the same growth rhythm, prevailing in nature. Retention of embryogenic potential after 3 years of culture holds tremendous importance in conservation, mass propagation, and successful genetic transformation. Embryogenic potential of somatic embryos for long is on record in a number of plant species but did not exceed more than two years (Kaur et al., 2006; Sahrawat and Chand, 2001). Growth performance evaluation of somatic embryo derived cormlets under greenhouse conditions indicated dependence of percent sprouting upon cormlet size. A perusal of literature reveals that there is no report on growth performance evaluation of somatic embryo derived in vitro cormlets under green house conditions which is an important aspect of the present findings. The in vitro cormlets can be stored and transported easily for plantation in the favorable season and used efficiently for field transfers. Moreover the laborious step of acclimatization is eliminated and high percent survival can be achieved.

The involvement of epidermal (Fig. 5d) and hypodermal regions (Fig. 5e) of leaf in somatic embryogenesis was observed and has earlier been reported in different species (Mandal and Gupta, 2003; Yamamoto et al., 2005). The pockets of cells (4–6) enclosed by a common cell wall indicated unicellular origin of proembryo-like structures. These pockets of cells (Fig. 5e) are characteristic of early embryogenic events preceding embryo formation in the direct somatic embryogenesis (Kurczyjska

Fig. 4. In vitro cormlet production a) from somatic embryo derived shoots; b) Harvested cormlets (0.6–0.8 g); c) in vivo produced corms (0.6–0.7 g).

Table 6
Growth performance evaluation of cormlets generated through somatic embryo derived shoots.

Weight range of cormlets	Mean number of sprouts (SEm = 0.36)	Mean number of leaves (SEm = 0.19)	Mean length of leaves (cm) (SEm = 28.35)	% sprouting after 3 months
<0.05	1.00 ± 0.00^{b}	1.00 ± 0.00^{b}	7.75 ± 5.29^{b}	50.0
0.07–0.09	1.25 ± 0.25^{ab}	1.00 ± 0.00^{b}	7.63 ± 3.53^{b}	54.45
0.1–0.2	1.33 ± 0.33^{ab}	1.33 ± 0.33^{ab}	13.17 ± 2.52^{ab}	63.63
0.2–0.3	1.27 ± 0.19^{ab}	1.45 ± 0.16^{ab}	15.27 ± 1.78^{ab}	78.94
0.3–0.4	1.33 ± 0.33^{ab}	1.67 ± 0.30^{ab}	16.57 ± 0.91^{ab}	75.0
0.4–0.6	1.00 ± 0.00^{b}	2.00 ± 0.0^{a}	19.13 ± 2.18^{a}	80.0
0.6–0.7	2.00 ± 0.50^{ab}	1.67 ± 1.00^{ab}	18.19 ± 0.34^{ab}	79.0
0.7–0.8	2.25 ± 0.50^{a}	1.63 ± 0.25^{ab}	16.65 ± 0.13^{ab}	81.0
1.0–1.2	1.0 ± 0.20^{b}	2.00 ± 0.00^{a}	13.13 ± 1.66^{ab}	89.0

Data represented as means of 15 cormlets; data recorded after 5 months of transfer; values followed by lower case letters differ from each other at $p \leq 0.05$ (Duncan's multiple range test).

et al., 2007). Globular embryos (Fig. 5g) were covered with a well defined protodermis which is a characteristic feature of somatic embryo development (von Arnold et al., 2002; Yeung, 1995). A notch at the terminal position of the somatic embryo with scutellum and coleoptiles (Fig. 5h, i) was reported in ginger (Lincy et al., 2009) and *Cymbopogon pendulus* (Bhattacharya et al., 2010). Some of the somatic embryos had single root and number of shoot primordia (Fig. 5k). Simultaneous development of shoot, root and corm did not appear as reported earlier

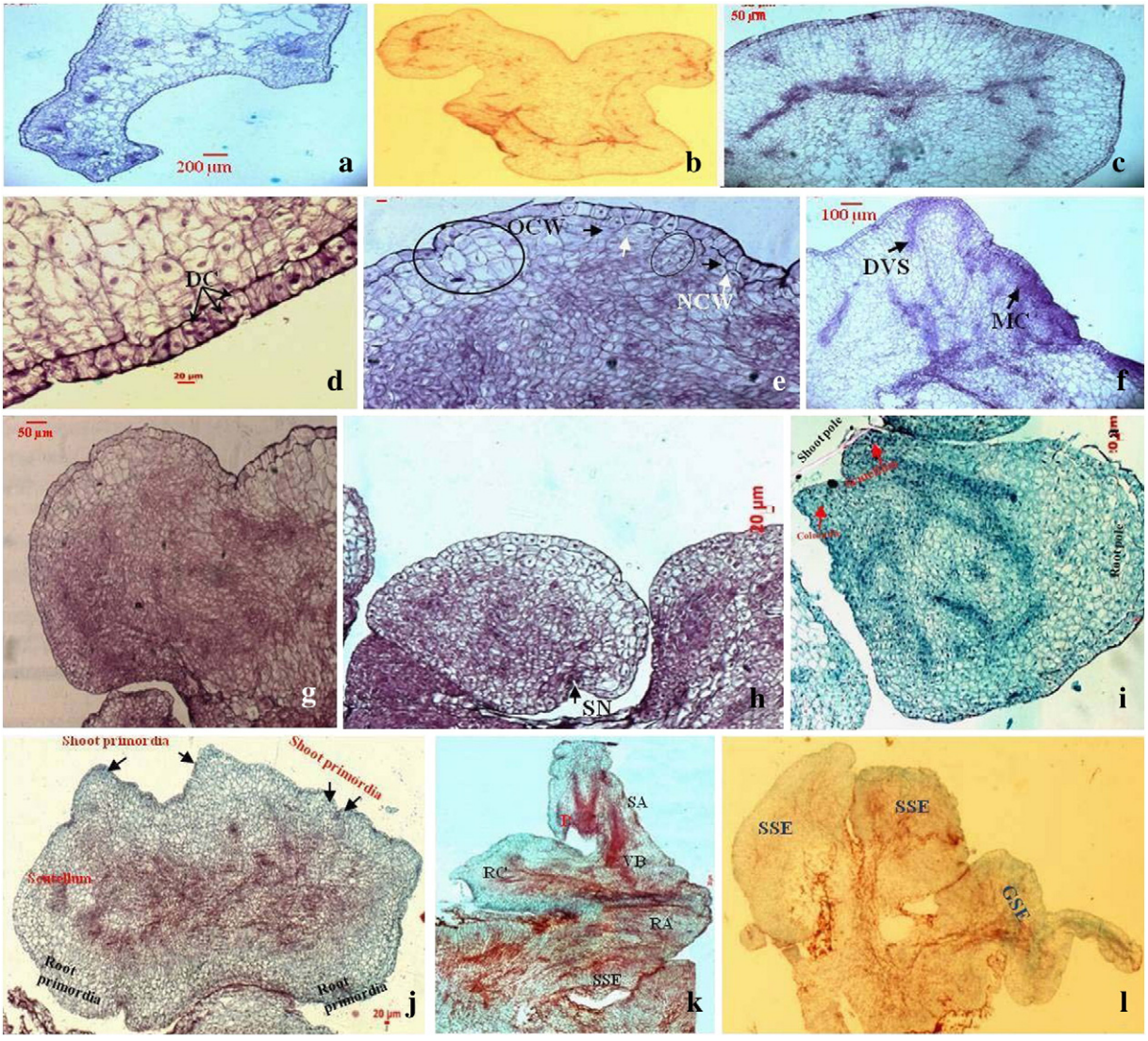

Fig. 5. Histological studies a) TS of leaf at day zero; b) swollen leaf after 4 wks on picloram (2.0 µM) and TDZ (2.5 µM); c) swollen leaf with unorganized vascular bundles; d) cell division in leaf epidermal cells, (DC — dividing cells); e) cell division in hypodermal cells, 4–6 celled structures enclosed by common thick wall (OCW — old cell wall, NCW — new cell wall); f) protuberance emerging from surface of leaf, (DVS — developing vascular bundle, MC — meristematic cells; g) pro-globular embryo; h) somatic embryo with scutellar notch (SN); i) scutellar embryo with shoot pole, root pole, scutellum and independent vascular system; j) somatic embryo germinated to form multiple shoot primordia; k) germinated somatic embryo with shoot apex (SA), root apex (RA), roundish corm (RC) and prophyll (P), vascular bundle (VB) and secondary somatic embryo (SSE) at the base; l) secondary (SSE) embryogenesis from the base of germinated somatic embryo (GSE).

Table 7
Biochemical estimation of somatic embryos matured in EPM supplemented with different concentrations of ABA.

mg g^{-1} fw	Control	ABA (μM)			
Parameters (15 days of treatment)		1.89	3.78	7.56	15.12
Amylase	0.30 ± 0.01	0.28 ± 0.02	0.30 ± 0.05	0.28 ± 0.003	0.29 ± 0.02
Starch	1.27 ± 0.01	2.64 ± 0.01	2.47 ± 0.00	4.40 ± 0.01	3.62 ± 0.02
TSS	9.23 ± 0.10	20.08 ± 0.004	11.28 ± 0.02	34.75 ± 0.04	34.10 ± 0.001
RS	6.79 ± 0.02	3.63 ± 0.02	6.50 ± 0.04	5.14 ± 0.01	8.40 ± 0.04
NRS	2.78	16.63	5.11	29.86	26.12
30 days of treatment					
Amylase	0.25 ± 0.27	0.25 ± 0.27	0.27 ± 0.28	0.28 ± 0.29	0.25 ± 0.27
Starch	0.91 ± 0.004	0.83 ± 0.003	0.26 ± 0.002	1.93 ± 0.01	1.62 ± 0.004
TSS	27.88 ± 0.01	34.41 ± 0.02	27.41 ± 0.03	40.21 ± 0.02	42.75 ± 0.01
RS	12.62 ± 0.01	9.47 ± 0.01	23.97 ± 0.003	5.87 ± 0.001	14.24 ± 0.02
NRS	15.89	25.42	4.64	34.64	29.22

Data given are means of 3 replicates and represented as mean ± standard error.

by Bhattacharya et al. (2010) in *Cymbopogon pendulus*. Surface cells of globular embryos were smooth as reported in other plants (Bandyopadhyay and Hamill, 2000) and regularly arranged. Stomata were also observed (Fig. 2b) in some of the somatic embryos. In *Coffea arabusta*, stomata were observed in precotyledonary and germinated stage of somatic embryo (Afreen et al., 2002).

Storage of reserve accumulation is an important marker of physiological state and it may affect the development of somatic embryos and conversion into plantlets (Cailloux et al., 1996). Somatic embryogenesis and its conversion require high amounts of energy, which is provided in the form of ATP by hydrolysis of starch accumulated during maturation phase (Martin et al., 2000). Starch may serve as a temporary carbon storage reserve to be used in biosynthesis of other storage materials like oil (Norton and Harris, 1975) or oligosaccharide (stachyose) (Yazdi-Samadi et al., 1977). In the present study, increase in starch content was related to the concentration of ABA (Table 7) but decreased with prolonged culturing for 30 days. Despite the role of ABA in storage accumulation, a promoting effect was not observed in the conversion of saffron somatic embryos.

In somatic embryos matured in ½ EPM, starch (5.51 mg g^{-1} fw) and TSS (38.5 mg g^{-1} fw) content as well as amylase activity (10.81 mg g^{-1} fw) were high and decreased in early germinated somatic embryos in MS basal medium (Table 7). During germination, decrease in amylase activity is reported in alfalfa seeds (Kohno and Nanmori, 1992) as amylase activity is related to decline of starch as reserve (Kepczynska and Zielinska, 2006). Godbole et al. (2004) also reported abrupt decline in amylase activity at embling stage in *Dendrocalamus hamiltonii*.

High percent germination (60.48) in somatic embryos matured in ½ EPM compared to those in EPM supplemented with ABA (30.0) was validated with higher reserve accumulation. Reports on ineffectiveness of ABA in somatic embryo maturation and conversion are available in literature (Mondal et al., 2002). Sharma et al. (2004) hypothesized that low reserve accumulation results in poor or no germination of tea somatic embryos.

In conclusion, direct somatic embryogenesis offers a viable protocol for saffron propagation with little chances of variations. TDZ and picloram in varied concentrations were effective for induction and proliferation of somatic embryos, whereas maturation was favored in ½ EPM. Retention of embryogenic potential beyond 3 years of culture holds tremendous importance in mass propagation, conservation and genetic transformation studies. This is an important approach other than organogenesis for eventual cormlets production in a commercially important crop like saffron.

Table 8
Biochemical analysis of somatic embryos matured in ½ EPM with 3.0% sucrose and germinated in MS basal medium.

Parameters (mg/g fw)	Proliferating somatic embryos	Matured somatic embryos	Germinated somatic embryos
Amylase	9.50 ± 0.47	10.81 ± 0.51	2.61 ± 0.06
Starch	3.97 ± 0.43	5.51 ± 0.81	4.83 ± 0.23
TSS	23.09 ± 0.21	38.5 ± 0.71	11.18 ± 0.52
RS	6.02 ± 0.12	3.87 ± 0.17	2.33 ± 0.73
NRS	17.07 ± 0.19	34.62 ± 0.73	9.64 ± 0.43

Data given are the means of 3 replicates and represented as mean ± standard error.

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